The Immunogenicity of a Biological Simulant: Strategies for the Improvement of Antibody-Based Detection

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Abstract: When biological simulants are used for testing detection/identification instruments, antibodies are often employed to quantitate the amount of simulant or sometimes as an integral part of the detection suite. The bacterium *Pantoea agglomerans* (formerly *Erwinia herbicola*, *Eh*) presently is used to simulate vegetative biological agents, however, anti-*Eh* antibodies of high affinity and specificity are needed. Therefore, we have characterized rabbit antibodies raised against native *Eh* and *Eh* chemically modified with dinitrophenyl groups (*Eh*-DNP) as a strategy to increase intrinsic *Eh* antigenicity. Our second strategy is to genetically manipulate *Eh* cell surface components so that we can employ commercially available antibodies or potentially antibodies to epitopes of known agents displayed on *Eh*. Here we report progress on characterization of anti-*Eh* antibodies by ELISA and Western blot analyses, and on expression of foreign genes in *Eh* in developing the surface display approach. Anti *Eh*-DNP preparations contained antibodies against the DNP label, as expected, and were equally or slightly more efficient in detecting native *Eh* cells, compared with antibodies raised against native *Eh*. Affinity purification procedures significantly improved *Eh* detection sensitivity. Both ELISA and Western blot analyses showed marked cross reactivity against other bacteria such as *E. coli*. This finding emphasizes the need for an alternative approach for specific detection. We have successfully transformed *Eh* with several different vectors and have cloned and sequenced a prominent *Eh* outer membrane protein needed for display of specific polypeptide tags. Ultimately this should extend *Eh* applicability as a simulant expressing specific markers from selected agents.

Introduction

Erwinia herbicola is a rod-shaped, non-pathogenic, Gram-negative facultative anaerobe, belonging to the family Enterobacteriaceae, and as such is an appropriate simulant for *Yersinia pestis* and other Gram-negative pathogens (e.g., *Francisella tularensis*). *E. herbicola* has been approved for environmental release at Dugway Proving Ground, and at present is the common simulant in tests requiring vegetative bacteria. (It is important to note that the accepted scientific name of the strain used has been changed to *Pantoea agglomerans* ATCC 33243, however we will use *Erwinia* or *Eh* synonymously here, as the strain is commonly termed this way at ECBC and this designation has been used in reports.) *Eh* according to the testers of equipment is presently deficient in two ways. The first is that its antigenicity is not adequate as a

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backup for other identification procedures being used. The second is that the survivability of *Eh* is quite low when it is aerosolized to mimic an aerosol threat (1,2). The aspect of survivability is currently under investigation by others at the Aerosol Science group at the ECBC.

In the present study we report results of efforts to obtain improved antibody-antigen responses in order to facilitate advances in the use of *Eh* as a simulant. This report is intended to provide a brief overview of the outcome of these studies, with a more detailed description to be published elsewhere. The results presented here relate for the most part to the first of two-approaches being applied to enhance the antigenicity of *Eh*, that of a chemical labeling strategy to modify *Eh* cell surface epitopes.

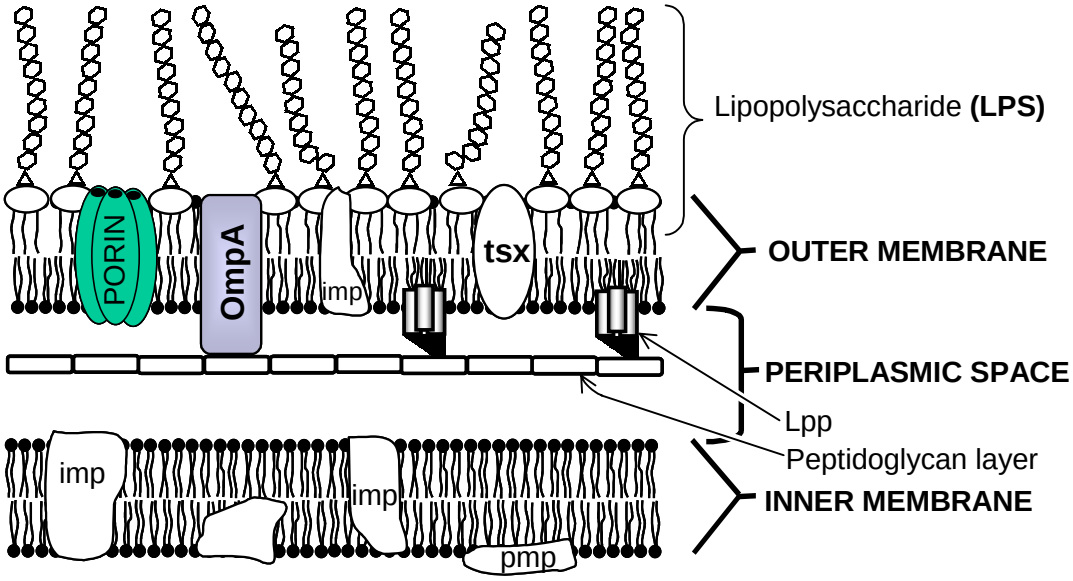
The chemical modification approach is based on the fact that introduction of chemical groups in the vicinity of a weakly antigenic structure results in antibody production not only against the newly attached chemical group, but also often elicits a stronger immunological response against the native structure as well. This property was previously pointed out to one of us (B.V.B.) at an earlier meeting by Dr. Jose-Luis Sagripante, see also (3). In the present study, the results from quantitative ELISA analyses of antibodies raised against dinitrophenyl-modified *Eh* cell envelope preparations are compared with those obtained from antibodies raised against the native membrane components.

Some preliminary results are also reported here for the second approach, wherein we are applying molecular biological/molecular genetic techniques to introduce specific oligopeptide tags on the surface of *Eh*, which is expected to result in significant enhancements of antigenicity and specificity of detection. The method of expressing short oligopeptide tags on an outer membrane protein present in high abundance on the cell surface of Gram-negative organisms (e.g., the abundant, outer membrane protein, OmpA) is potentially applicable to many bacterial species. Since the approach is not limited to *Eh*, we expect it should be adaptable to a wide variety of Gram negative bacteria, including those that may be of interest for future development as vegetative bacterial simulants. This system would then allow the use of commercially available antibodies for simple, inexpensive, and highly specific detection during field tests. Furthermore, since a wide array of peptide structures can be chosen for expression, the approach ultimately should be able to imitate exterior epitopes of specific pathogenic agents (e.g., *Yersinia pestis*), so that more realistic but still safe tests may be performed.

Results and Discussion

Chemical modification strategy

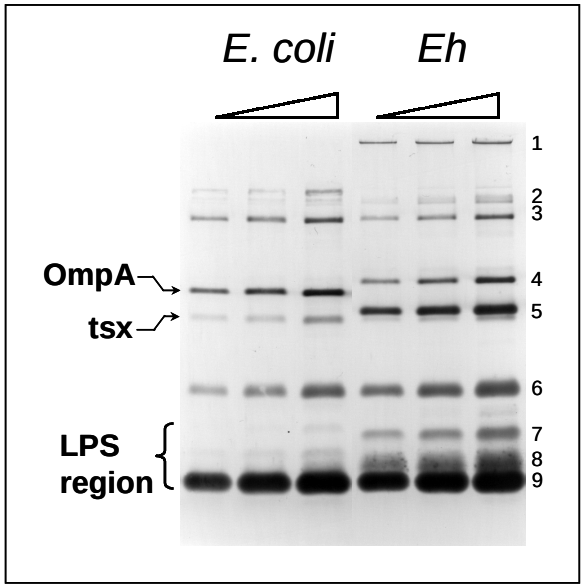
The Gram-negative enterobacterial cell envelope, shown schematically in Fig. 1, contains many different complex lipid, carbohydrate and protein components that can interact with the external environment, and thus are of potential use for the development of detection strategies (4). Rabbit anti-*Eh* antibodies already available from the ECBC Critical Reagents Repository (CRP) had until now not been characterized by Western blot analyses, and out initial experiments showed that these antibodies react with a number of such components, both proteins and polysaccharides, as shown in Fig. 2.



imp and pmp, integral and peripheral membrane proteins; Lpp, murine lipoprotein; OmpA, outer membrame protein A; tsx, nucleoside specific channel protein

Figure 1. Structure of the Gram-negative enterobacterial cell envelope (4).

Moreover, and perhaps not too surprisingly, the available antibodies showed marked cross reactivity with similar components on other bacteria such as *E. coli*.



An SDS-PAGE gel was loaded with equivalent, increasing levels of extracts from *E. coli* and *E.herbicola*.

The gel was electroblotted on a PVDF membrane, and probed using ECBC antibody preparation 220997-01 at a 1:5000 dilution. Colorimetric detection was performed using HRP-conjugated secondary antibody.

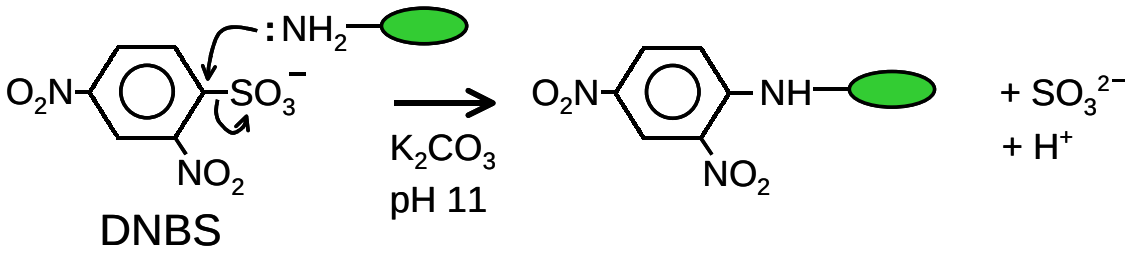
E. coli protein bands 4 and 5, cut out in a separate run, were used for N-terminal sequencing.

Sequence results identified band 4 as OmpA (outer membrane protein A, 36 kDa), and band 5 as tsx (a 31 kDa outer membrane nucleoside specific channel), as two of the strongly cross-reacting components recognized by the ECBC antibodies.

1º Ab: ECBC 220997-01 Rabbit anti-*E. herbicola* 1:5000
2º Ab: Goat anti-rabbit IgG HRP conjugate

Figure 2. Western blot analysis of whole cell extracts from *E. coli* and *E. herbicola*.

Chemical labeling with DNP was performed on whole cells of *Eh*, by a method similar to that used previously for *E. coli* (5), by exposure of the cells to the reagent 2,4 dinitrobenzenesulfonic acid (DNBS), as outlined in Fig. 3. Following the labeling reaction, a crude outer membrane fraction was isolated and used for immunization of rabbits. Three different crude cell envelope preparations were used in a standard immunization protocol carried out in three separate animals, in which Freund's complete adjuvant was included for initial injections, but not for subsequent boosts. Since DNP modification of amino groups by DNBS takes place under somewhat alkaline conditions, a control incubation lacking DNBS was also set up at the same pH. In another control, a cell envelope preparation was prepared closer to neutral pH by lysis of the cells with lysozyme. As indicated in Fig. 3, the chemically modified preparation is termed "Alk DNP" while the controls are designated "Alk" and "Neutral".



Three crude cell envelope preparations used for Ab production in rabbits:

1. K_2CO_3 + DNBS "Alk DNP"
2. K_2CO_3 – DNBS "Alk"
3. Lysozyme lysed cells (pH 8) "Neutral"

Figure 3. DNP labeling of *E. herbicola* cells: cell envelope preparations used for rabbit antibody production.

Western blot analyses were performed using antisera obtained against each of the preparations, and the results are shown in Fig. 4. As expected, strong antibody reactivity against the DNP hapten itself was found in the case of antibodies raised against DNP-labeled *Eh* outer membrane preparations, as shown by reaction with DNP-conjugated human serum albumin (DNP-HSA) (left panel, third lane). And, no reaction with DNP-HSA was seen for control antibodies (center and right panels, third lanes). In order to quantify the response, purified IgG preparations were prepared from batches of antisera obtained from each animal using protein A-Sepharose. Purified IgG was then used in ELISA analyses for detection of *Eh* cells adsorbed to standard 96-well plates. As shown in Fig. 5 (top), the sensitivity for detection of *Eh* was roughly similar for all three preparations, and differed little from the rabbit anti-*Eh* previously obtained from CRP at ECBC. Certain minor differences in these preparations were noted, e.g., the Alk DNP

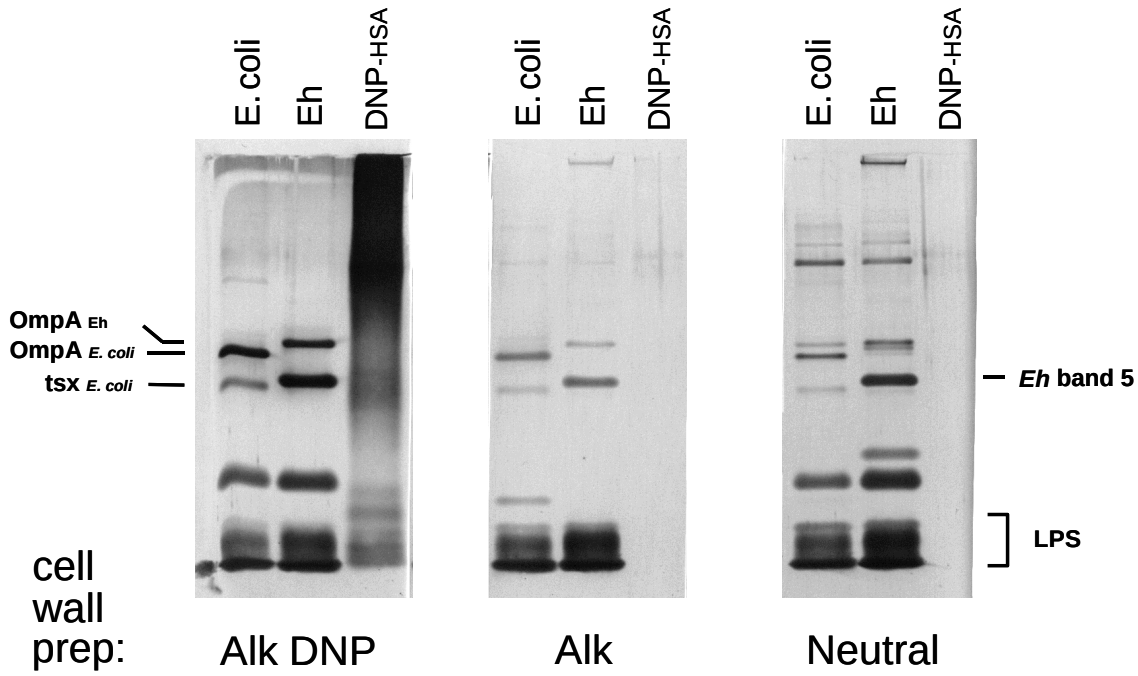
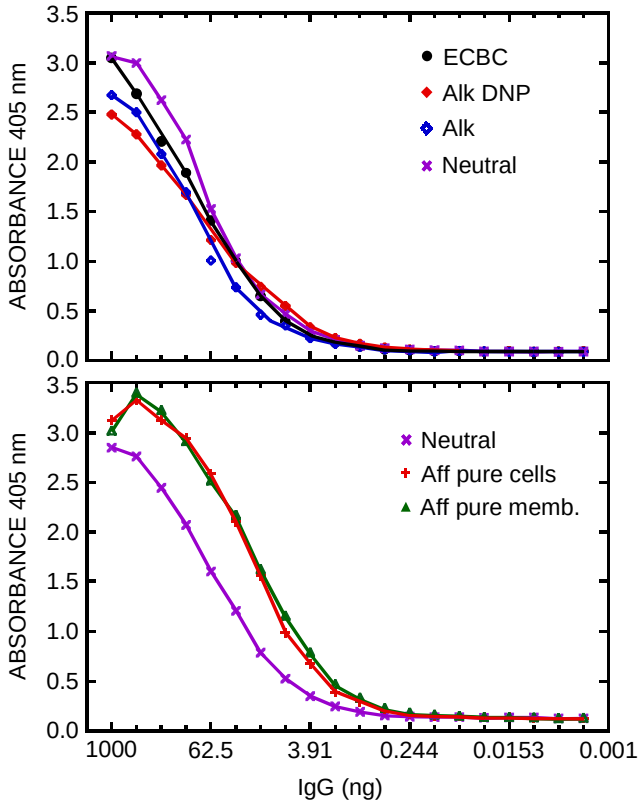


Figure 4. Analysis of rabbit antibodies raised against different Eh cell wall preparations by Western blots.



ELISA ANALYSES

Roughly equal responses were found for antibodies raised against the different Eh cell envelope preparations.

However, Ab affinity purification gave ~ 4X higher detection sensitivity.

Figure 5. Quantitative ELISA analyses of anti-*Eh* antibodies (IgG fractions) raised against different *Eh* cell envelope preparations

antibodies consistently exhibited a more shallow curvature of the plots, indicating that they comprise a different population of antibodies relative to the other preparations. The response of the Alk DNP antibodies was such that somewhat higher sensitivity was obtained, and this was most significant at levels of IgG that were lowest in the titrations. This indicated that at least a portion of the Alk DNP antibody population does exhibit an altered, more sensitive response. However, overall the comparison shows, that without modification of the assay or further purification, the Alk DNP preparation is not likely to provide markedly better detection than other antibodies raised against unlabeled *Eh*.

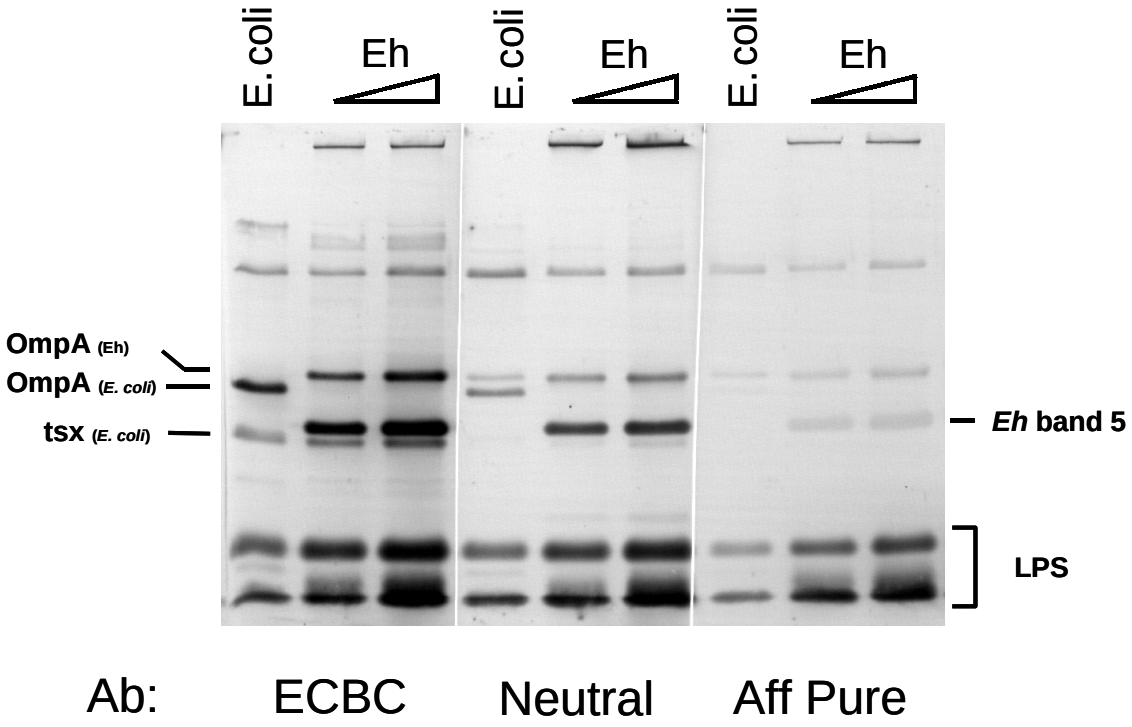


Figure 6. Detection of a different set of *Eh* antigens following Ab affinity purification

In order to increase detection sensitivity, a simple method was developed to further purify the Eh antibodies. Anti-*Eh* IgG preparations were incubated with a suspension of *Eh* to allow antibody binding to the cells, washed, and thereafter released from the cells by treatment at pH 3.5. After removal of the cells by centrifugation, the released antibodies were then brought to neutral pH, captured on protein A Sepharose and reconcentrated. As shown in Fig. 5 (bottom) this afforded an approximately 4-fold increase in sensitivity. A membrane-based method for immobilization of *Eh* was also used, with similar results. Characterization of the affinity purified antibodies by Western blot analysis showed that the procedure resulted in selection of a sub-population of the original antibodies. Notwithstanding the fact that the affinity purified antibodies showed a markedly increased sensitivity for *Eh*, a different set of *Eh* antigens was detected following affinity purification, as shown in Fig. 6. Notably, a number of protein components were not well recognized by the affinity purified antibodies, suggesting that the most highly immunogenic epitopes of these proteins are not accessible on the cell

surface, and may reside inside the membrane spanning regions or within the periplasm. By contrast, anti-LPS antibodies were well represented in the affinity purified preparations (Fig. 6), suggesting that the procedure resulted in an enrichment of those antibodies recognizing LPS structures. Furthermore, the results indicate that the 4-fold increase in sensitivity for *Eh* found in the ELISA format primarily involves detection of antibodies bound to lipopolysaccharides. Currently we are exploring gentle alternative chemical methods for specific labeling of LPS components with the goal of developing new procedures that would take maximum advantage of the high sensitivity and specificity of chemical modification, with minimum adverse effects on the native cell surface structure.

Genetic cell surface modification strategy — preliminary results

With the exception of one of the major bands seen in the Western blots, i.e., *Eh* band 5, antibodies raised against *Eh* are generally non-specific, and cross react relatively strongly with a number of similar components in the outer membrane fractions from other bacteria, such as *E. coli*. This non-specific cross reactivity decreases the reliability of detection that can be expected using these antibodies, and also ultimately limits their detection sensitivity at the point at which background levels in environmental samples become significant due to the presence of other cross-reacting organisms. Therefore, a method was sought that would be highly sensitive as well as providing a high specificity for detection of *Eh*. Ideally, any such method also would be readily modifiable for future uses of *Eh* carrying agent-specific markers for wider simulant applications.

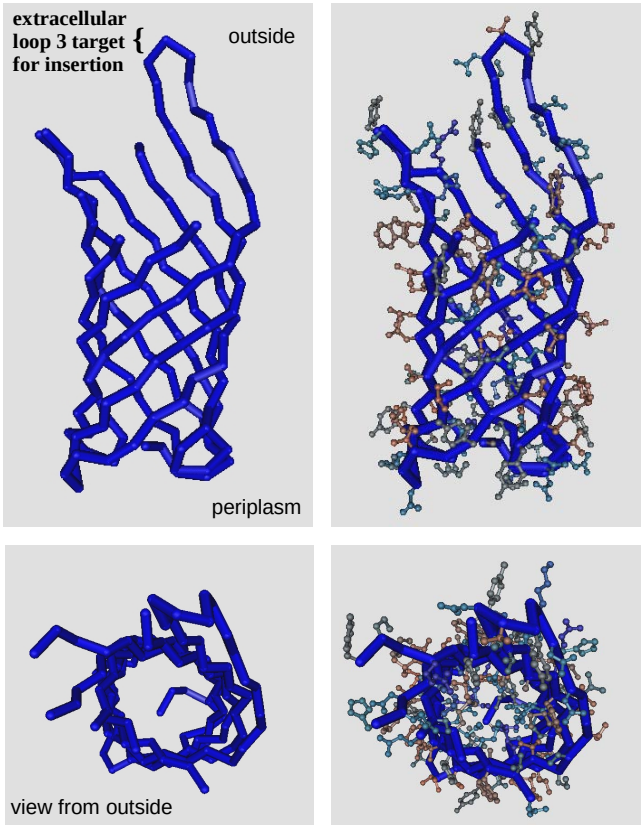


Figure 7. Analysis of OmpA transmembrane structure (8) in the strategy to display polypeptide tags on the *Eh* outer membrane.

A survey of the literature together with our results to date indicate that the highly abundant outer membrane protein OmpA, which functions to stabilize the outer membrane and is required for efficient F-dependent conjugation, would be a good candidate for use in a system for surface expression of chosen peptides (6,7). Such foreign peptide tags exposed on the cell surface, carried by OmpA, would be readily detectable with high sensitivity and specificity using available anti-peptide antibodies. The ~ 35 kDa outer membrane protein OmpA acts a monomer and, as shown in Fig. 7, consists of 8 transmembrane spanning segments arranged in a β barrel connected by loops (8). Four of these loops are relatively large and

are exposed on the extracellular surface. *E. coli* OmpA mutants have been studied in which these loops have been either lengthened or shortened without deleterious effects on the overall protein structure, membrane stability, or assembly of OmpA in the membrane (9-11). Therefore, our strategy is to generate OmpA mutants containing specific peptide insertions, ranging in length from 10 to 30 amino acids, in a selected extracellular loop(s). Cloning and sequencing *Eh* OmpA is a prerequisite for generating the desired peptide insertion constructs, therefore, we have carried out PCR amplification of a 873 bp region of the *Eh* OmpA gene and sequenced this portion of the gene. Figure 8 shows the

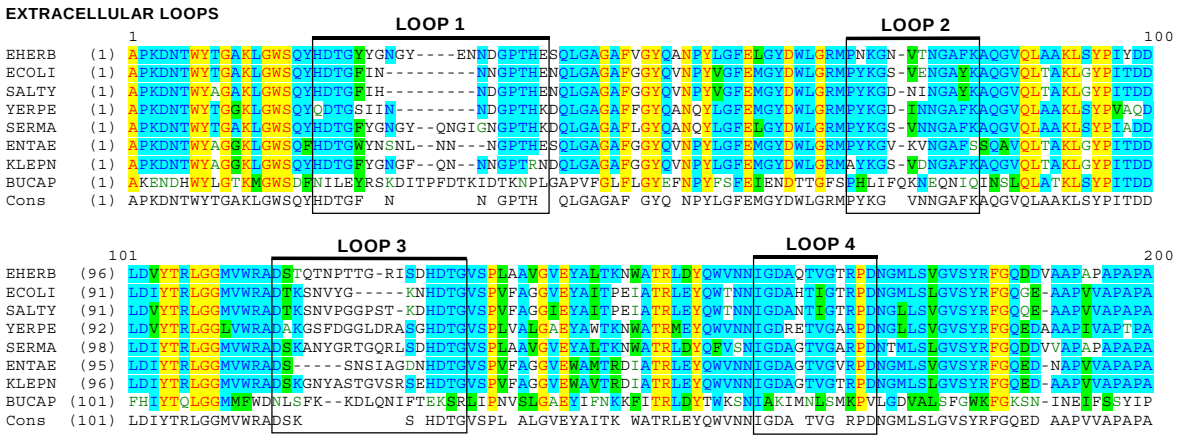
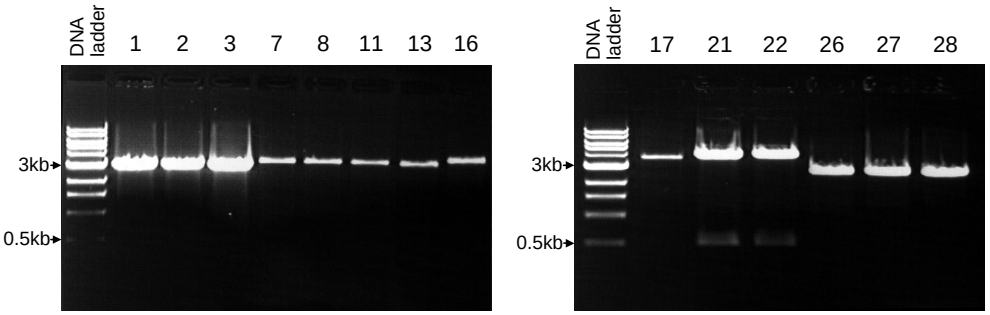


Figure 8. The amino acid sequence (standard single letter code abbreviations) of *Eh* OmpA determined here is aligned with other enterobacteria over a region of 200 amino acids that contains extracellular loops 1-4, showing characteristic sequence variability among the different organisms. EHERB, *Erwinia herbicola* ATCC strain 33243 (this work); ECOLI, *Escherichia coli*; SALTY, *Salmonella typhimurium*; YERPE, *Yersinia pestis*; SERMA, *Serratia marcescens*; ENTAE, *Enterobacter aerogenes*; KLEPN, *Klebsiella pneumoniae*; BUCAP, *Buchnera aphidicola*; Cons, consensus.

amino acid sequence of *Eh* OmpA in the region encompassing the four extracellular loops aligned with other organisms representing diverse enterobacterial genera. The results show the highest degree of sequence variability in loops 1 and 3, identifying these loops as good candidates for peptide insertion. We also have used the PCR amplified OmpA fragment as a probe for a Southern blot with Eh genomic DNA digested with different restriction enzymes. The results, Fig. 9, show a number of genomic fragments that hybridize with the *Eh* OmpA PCR probe. One such example is a 7.5 kb EcoRI fragment containing the *Eh* OmpA gene, that we have now cloned in order to obtain complete sequence information including the native promoter.

Since molecular biological techniques have not been established in *Eh*, it was essential for us to prove that this wild type organism could be made competent for transformation with useful plasmid vectors for cloning and expression. As shown in Fig. 10, *Eh* could be transformed with a variety of plasmids, which were taken up and maintained. In addition, we also showed that *Eh* expresses foreign plasmid-encoded proteins following transformation with the plasmid pEGFP (Clontech, BD Biosciences), which encodes a modified form of green fluorescent protein from the jelly fish *Aequorea victoria* (Fig. 11). The availability of fluorescent *Eh* could be useful as a simple means for locating cells in certain field or chamber experiments.



Erwinia herbicola cells were made competent by the standard calcium chloride method, and were transformed with different plasmids. Following transformation, cells were plated on TSB plates containing antibiotics appropriate for the antibiotic resistance markers contained on each plasmid. Numbers correspond to the designated colony picked from the plates. The individual colonies were used to inoculate 10 ml broth cultures for overnight growth and plasmid isolation. All plasmids have been linearized by restriction enzyme digestion prior to analysis on a 1% agarose gel stained with ethidium bromide. DNA ladder marker bands correspond to: 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10 kb

Transformants:

1, 2, 3	pBluescript II KS+ (Stratagene) = 2.96 kb
7, 8, 11, 13	pQE60 expression vector (Qiagen) = 3.43 kb
16, 17	pREP4 derivative of pACYC (Qiagen) = 3.74 kb
21, 22	pCRII-TOPO (PCR cloning vector, Invitrogen) = 3.9 kb + 0.6 kb insert
26, 27, 28	pUC18 = 2.69 kb

Figure 9. Transformation of *E. herbicola* with various plasmids.

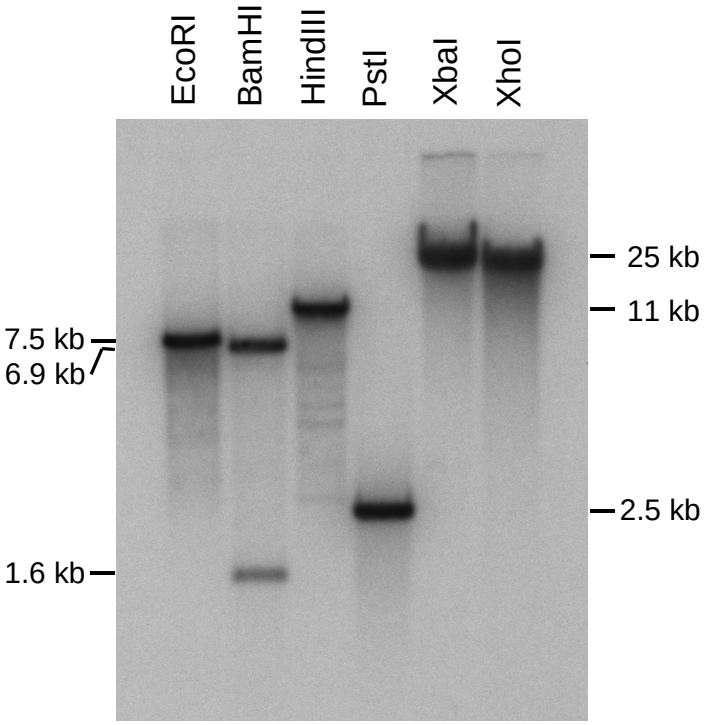


Figure 10. Southern Blot of *E. herbicola* DNA probed with an *Eh* OmpA PCR fragment

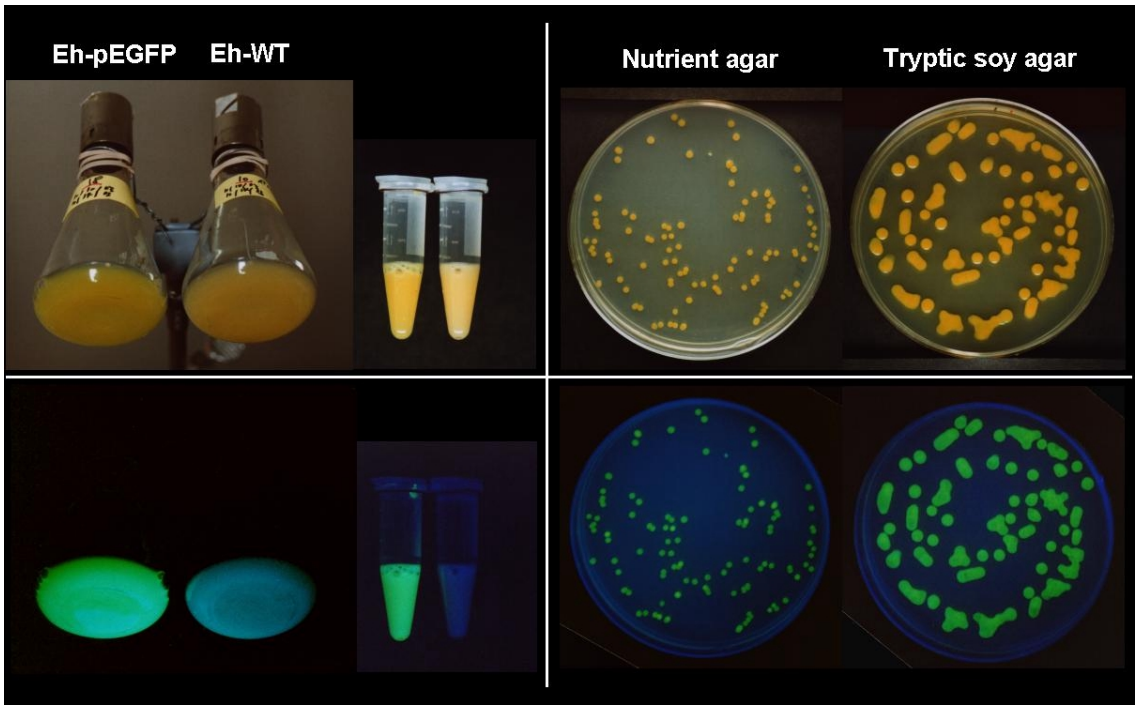


Figure 11. Demonstration that *Eh* can be used for expression of foreign proteins, e.g., green fluorescent protein from the jelly fish *Aequorea victoria*. Liquid cultures of *Eh* transformed with the plasmid pEGFP, containing the gene for green fluorescent protein, (Eh-pEGFP) and wild type cells (Eh-WT) were photographed under normal room light (left, top) and under long-wavelength UV light (left, bottom). *Eh* transformed with pEGFP, plated on nutrient agar and on tryptic soy agar, showed colony morphology differences typical for effects expected from growth on these different media (right, top), and all colonies exhibited green fluorescence under UV light (bottom, right).

Summary

Two avenues are being explored to increase the immunogenicity of *Eh* for further improvement of this organism as a vegetative bacterial simulant. The first, involving chemical modification of the cell surface with dinitrophenyl groups, yielded antibody preparations that were somewhat altered in their specificity and affinity for native *Eh*. However, at the present level of development these preparations are not likely to provide markedly better detection overall than other available antibodies, all of which suffer from general cross reactivity, as seen in Western blot analyses. An efficient affinity purification method provided a 4-fold increase in antibody sensitivity, and revealed a sub-class of antibodies recognizing *Eh* lipopolysaccharide surface components. At the present time alternative chemical approaches are being developed for modification of LPS carbohydrate components. The second avenue involves molecular biological techniques to produce a genetically modified form of OmpA that will serve as a carrier protein for expression of foreign oligopeptide tags of choice on the *Eh* cell surface. This is expected to lead to higher sensitivity of detection, greater detection specificity, and the

ability to mimic surface epitopes of specific threat agents. Preliminary results reported here show that *Eh* can be successfully transformed with a variety of different cloning and expression vectors, demonstrating the ability of *Eh* to maintain useful genetic vectors in high copy numbers. In one case, the use of pEGFP produced green fluorescent Eh cells, which could be useful as a simple means for locating cells in certain field or chamber experiments. The cloned *Eh* OmpA gene together with its native promoter region is being sequenced to prepare the insertion constructs needed for cell surface oligopeptide expression.

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